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CD4 Down-Modulation by Human Immunodeficiency Virus Type 1 Nef Correlates with the Efficiency of Viral Replication and with CD4$^+$ T-Cell Depletion in Human Lymphoid Tissue Ex Vivo

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The human immunodeficiency virus type 1 (HIV-1) Nef protein is an important virulence factor. Nef has several functions, including down-modulation of CD4 and class I major histocompatibility complex cell surface expression, enhancement of virion infectivity, and stimulation of viral replication in peripheral blood mononuclear cells. Nef also increases HIV-1 replication in human lymphoid tissue (HLT) ex vivo. We analyzed recombinant and primary *nef* alleles with highly divergent activity in different in vitro assays to clarify which of these Nef activities are functionally linked. Our results demonstrate that Nef activity in CD4 down-regulation correlates significantly with the efficiency of HIV-1 replication and with the severity of CD4$^+$ T-cell depletion in HLT. In conclusion, HIV-1 Nef variants with increased activity in CD4 down-modulation would cause severe depletion of CD4$^+$ T cells in lymphoid tissues and accelerate AIDS progression.

Human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS, replicates efficiently and continuously in infected subjects, even in the presence of a strong antiviral host immune response (8). One factor, important for efficient viral persistence and the development of AIDS, is the product of the accessory HIV-1 *nef* gene. Initially, it has been shown that simian immunodeficiency virus (SIV) carrying a deletion in *nef* replicates inefficiently and usually does not cause disease in rhesus macaques (17). Subsequently, several studies identified human subjects infected with *nef*-defective HIV-1 isolates (7, 18). In agreement with the findings in the SIV/macaque model, these individuals showed low viral loads and prolonged disease-free survival.

A variety of in vitro Nef activities might contribute to AIDS progression. Nef down-modulates cell surface expression of CD4, the primary receptor for HIV and SIV (9, 23). This Nef function could promote virion release, enhance incorporation of the Env protein into viral particles, prevent superinfection, alter T-cell receptor signaling, and impair CD4$^+$ helper T-cell function (2, 19, 25, 28). Nef also down-regulates major histocompatibility complex class I (MHC-I) cell surface expression, likely allowing HIV-1 to escape from host immune surveillance (6, 27). Furthermore, Nef increases virion infectivity and accelerates viral replication in peripheral blood mononuclear cells (PBMC) (5, 24, 29). Recently, it has been demonstrated that Nef also enhances HIV-1 replication and interleukin 2 responsiveness in human lymphoid tissue (HLT) ex vivo (12). Neither the molecular mechanisms that underlie these Nef functions nor how they are linked to each other is fully understood.

It has become clear that some in vitro activities of Nef, e.g., CD4 and MHC-I down-regulation, are functionally separable (21, 30). For other Nef functions, such as CD4 down-regulation and the increased HIV-1 infectivity and replication, a mechanistic link has been proposed (19, 25). However, the relevance of Nef-mediated CD4 down-regulation for enhanced infectivity and replicative capacity remains controversial.

We investigated systematically which in vitro Nef activities might be functionally linked. Our results demonstrate that *nef* alleles impaired in down-modulation of CD4 cell surface expression are still capable of enhancing virion infectivity. Conversely, increased viral infectivity for MAGI indicator cells did not correlate with the replicative capacity in PBMC or HLT. However, we found a highly significant correlation between CD4 down-regulation and the efficiency of HIV-1 replication both in PBMC culture and in HLT ex vivo. Our data suggest that these Nef functions might involve common molecular mechanisms and indicate an important role of Nef-mediated CD4 down-regulation in the efficiency of viral replication in lymphoid tissues and, consequently, in AIDS progression.

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MATERIALS AND METHODS

Nef variants analyzed. *nef* alleles were derived from a long-term nonprogressor (LTNP4), which shows no signs of disease and has undetectable viral RNA load despite more than 17 years of HIV-1 infection (13, 14, 22), and from the progressing patient P9 (4). Recombinants between LTNP4 *nef* alleles and the

highly active NA7 *nef* have been described previously (22). P9 *nef* alleles were derived from PBMC samples obtained over a time period of 14 years. During this time the patient showed a dramatic decrease in the number of $CD4^+$ T cells and progressed to AIDS. As described elsewhere (4), the amplified PCR products were cloned directly into the bicistronic pCG-Nef-internal ribosome entry site-green fluorescent protein (GFP) vector or used to introduce *nef* genes into the HIV-1 NL4-3 molecular clone. A mixture of primary *nef* alleles was cloned as a pool to ensure that the *nef* genes analyzed were representative for each time point (4).

Transfection and fluorescence-activated cell sorter analysis. Transfection of Jurkat T cells and flow cytometry analysis of CD4, MHC-I, and GFP reporter molecules in cells transfected with a bicistronic vector coexpressing Nef and GFP was measured as described previously (4). Notably, the level of CD4 or MHC-I expression (red fluorescence) was measured from aliquots of the same transfection as a function of green GFP fluorescence. Quantitation of CD4 or MHC-I down-regulation by Nef was performed as described elsewhere (4).

HIV-1 infectivity and replication. Virus stocks were generated by transient transfection of 293T cells, and replication and infectivity assays were performed as described earlier (4).

HIV infection of HLT ex vivo and analysis of viral cytopathicity. Human tonsils removed during routine tonsillectomies were used for culture set up within 5 h of excision. The tonsils were dissected into 2- to 3-mm^3 blocks and infected by inoculating each block with 3 μl of viral stock suspension derived from transfected 293T cells as described previously (10–12). Infection doses were normalized based on p24 content. Productive HIV-1 infection was evaluated by measuring the amount of p24 core antigen released into the medium as described previously (12). Efficiency of virus production in histoculture was evaluated by calculation of the total amount of HIV-1 p24 antigen produced by one tissue block in the course of 11 to 13 days of infection. To evaluate $CD4^+$ T-cell depletion, cells were mechanically isolated from control and infected tissue blocks, stained for CD3, CD4, and CD8, and analyzed by flow cytometry as described previously (11). We used the $CD4^+/CD8^+$ ratio as a measure for $CD4^+$ T-cell depletion, since productive HIV-1 infection of HLT ex vivo does not change the number of $CD8^+$ T cells (11).

RESULTS

We utilized a set of *nef* alleles derived from LTNP4 and several recombinants with the functional NA7 *nef* allele (22) to determine which in vitro Nef activities might be functionally linked. All *nef* alleles efficiently down-modulate MHC-I and enhance virion infectivity. However, they have highly divergent effects on CD4 cell surface expression and on viral replication in PBMC (4, 22). In the present study we investigated their effect on HIV-1 NL4-3 replication in HLT ex vivo. This system allows one to analyze the relevance of different HIV-1 Nef functions for viral replication and $CD4^+$ T-cell loss in a setting that requires no exogenous activation and largely maintains the complexity of cell populations and tissue cytoarchitecture found in vivo (10, 12). As shown in Fig. 1A, all viruses were capable of replicating in HLT, albeit with different efficiency. The control NA7 *nef* allele efficiently increased viral replication in histoculture. However, a point mutation of A56D in the NA7 Nef, which disrupts CD4 down-regulation (22), impaired NL4-3 replication to the level of *nef*-deleted virus [Fig. 1; NA7-(A56D]. Similarly, the LTNP4-91-NA7 and LTNP4-91B1 *nef* alleles, which do not down-modulate CD4, also did not stimulate viral replication in HLT. Notably, substitution of D56A in the LTNP4-91-NA7 Nef fully restored both CD4 down-regulation (22) and viral replication in histoculture (Fig. 1; LTNP4-A-NA7). Finally, changes of D56A and K174E in the inactive primary LTNP4-91B1 Nef, resulting in some gain of function in down-modulation of CD4 surface expression (22), also increased the replicative capacity in HLT ex vivo [Fig. 1, LTNP4-(A,E)]. These differences in virus production were consistently observed in tissues derived from six to eight donors (Fig. 1B). On average, the Δnef HIV-1 variant replicated with slightly higher efficiency than NL4-3 carrying the LTNP4-91-NA7 or LTNP4-91B1 *nef* allele (Fig. 1B). Consistent with a previous study (18), this observation suggests that a smaller genomic size is advantageous for HIV-1 replication.

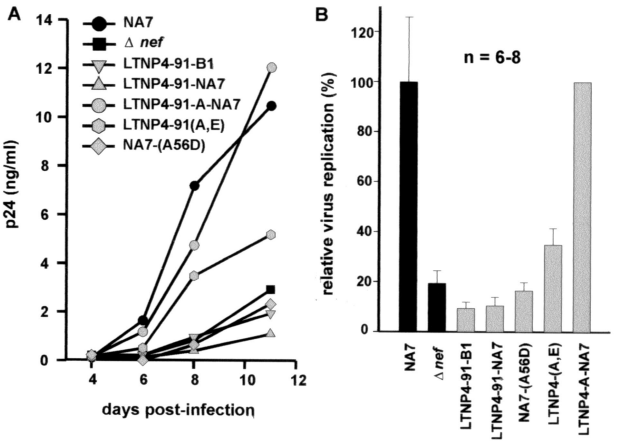

FIG. 1. Replication of HIV-1 NL4-3 Nef variants in HLT ex vivo. (A) Typical kinetics of p24 accumulation in the culture medium over an 11-day period. (B) Average amount of p24 released into culture media bathing tissues infected with NL4-3 *nef* variants over 11- to 13-day periods (means and standard errors of the means of tissues from six to eight donors).

Altogether, we have quantitated the activity of the LTNP4/NA7 *nef* alleles in five in vitro assays: (i) down-modulation of CD4; (ii) MHC-I down-regulation; (iii) enhancement of virion infectivity; (iv) stimulation of viral replication in PBMC; and (v) enhancement of virus production in HLT ex vivo. Subsequently, we assessed which of these activities are correlated. As demonstrated in Fig. 2, there is a significant correlation between Nef activity in CD4 down-regulation and the efficiency of virus replication in ex vivo infected HLT ($P = 0.0002$; Fig. 2A) and in human PBMC ($P = 0.02$; Fig. 2G). In agreement with these results, HIV-1 *nef* variants that replicated more effectively in PBMC also showed a higher replicative potential in lymphoid tissue ($P = 0.01$; Fig. 2D). Consistent with a previous report (30), Nef-mediated MHC-I down-modulation did not correlate with any other in vitro Nef activity investigated (Fig. 2B, E, H, and I). Notably, the enhancement of virion infectivity did not correlate with the efficiency of HIV-1 replication in PBMC or in lymphoid tissue (Fig. 2C and J). Furthermore, *nef* alleles impaired in CD4 down-modulation were still capable of increasing virion infectivity (Fig. 2F). These findings show that the effective enhancement of infectivity by Nef is insufficient for effective replication in primary T cells and that increased virion infectivity is not dependent on the reduction of CD4 cell surface expression.

Next, we tested whether the correlation between the efficiency of CD4 down-regulation and replication in HLT also exists for *nef* alleles obtained at different clinical stages of HIV-1 infection. The first PBMC sample was drawn in 1984 from P9, when the patient was clinically healthy and the number of $CD4^+$ T cells was within a normal range (>600/mm^3).

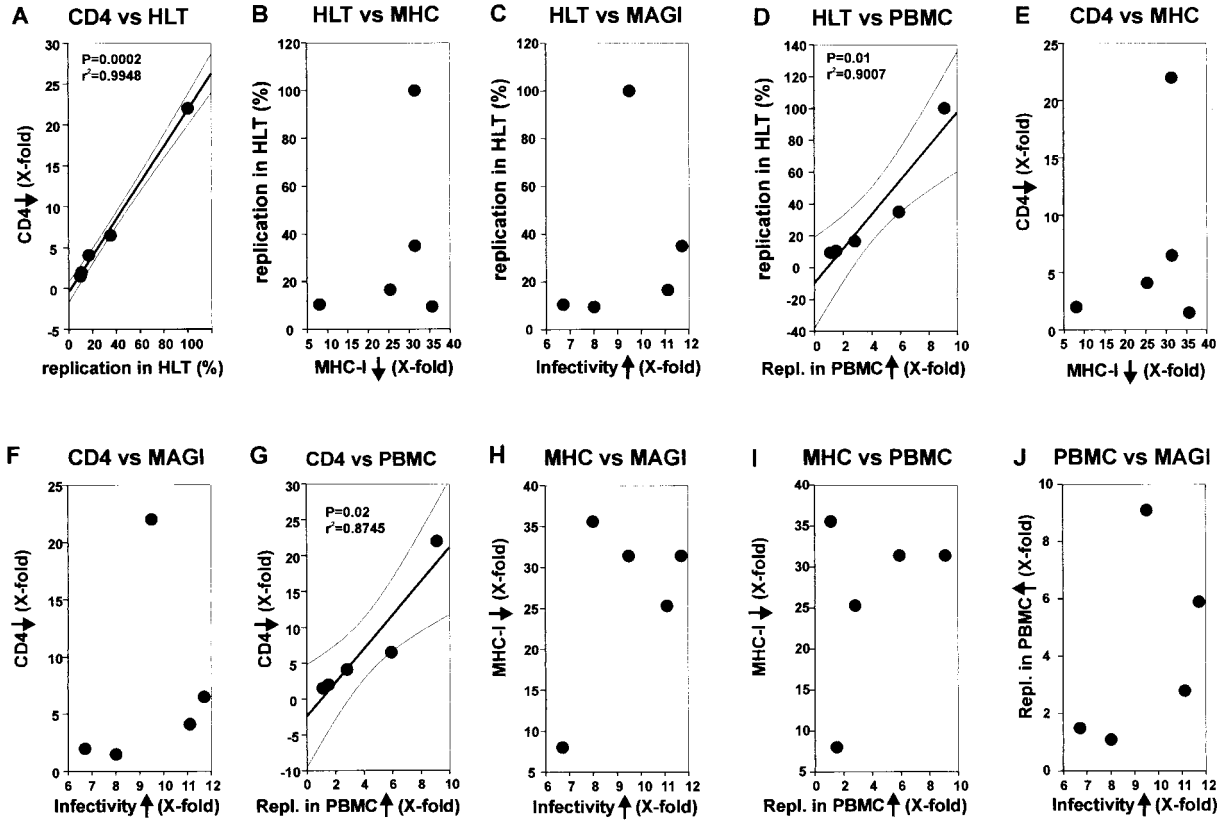

FIG. 2. Correlation between different in vitro Nef functions. The *nef* alleles derived from LTNP4 and different chimeric constructs containing portions of *nef* alleles from LTNP4 and NA7 (22) were investigated for their activity in five in vitro assays: (i) replication in HLT; (ii) CD4 down-regulation; (iii) down-modulation of MHC-I; (iv) enhancement of replication in PBMC; and (v) enhancement of virion infectivity. Functional assays were performed as described previously (4). Values for CD4 (at medium GFP expression levels) and MHC-I (at high GFP expression levels) down-regulation were obtained from 3 to 6 independent experiments. Each data point represents functional activity in two in vitro assays. For clarity, standard errors are not shown. Correlation coefficients and *P* values are indicated for functions that showed significant correlation.

The following samples were obtained during (1989; 118 $CD4^+$ T cells/mm^3) and after (1998; 95/mm^3) AIDS progression. Primary *nef* alleles were inserted into the respective vectors as a pool to ensure that they were representative for each time point (4). *nef* alleles derived from the 1989 and 1998 samples did not efficiently down-modulate MHC-I. In contrast, *nef* alleles amplified from the 1984 PBMC sample caused efficient reduction of MHC-I cell surface expression but showed reduced activity in down-regulation of CD4 (4). On average, the late P9 *nef* alleles, which were more active in CD4 down-regulation, also exhibited two- to threefold higher levels of replication in HLT ex vivo (Fig. 3). Although these differences varied to some extent between tissues from different donors, they were consistently observed and statistically significant (Fig. 3).

Our data demonstrate that Nef activity in CD4 down-regulation correlates with the efficiency of HIV-1 replication in HLT ex vivo. It has been suggested earlier that the extent of $CD4^+$ T-cell depletion in HLT ex vivo depends on the efficiency of HIV-1 replication (12). We infected tissues derived from multiple donors with different doses of the parental HIV-1 NL4-3 clone to confirm these findings. The efficiency of viral replication in different tissues varied considerably in a donor- and dose-dependent manner. We found a highly significant correlation between the efficiency of HIV-1 replication and $CD4^+$ T-cell depletion in all tested tissues ($P = 0.0001$; data not shown). Thus, Nef activity in CD4 down-regulation should also correlate with $CD4^+$ T-cell depletion in HLT. To verify this, we infected lymphoid tissues derived from two different donors with HIV-1 NL4-3 containing the sequential P9 or LTNP4/NA7 *nef* alleles, respectively. The results show that the depletion of $CD4^+$ T cells was relatively mild in tissues infected with HIV-1 NL4-3 containing *nef* alleles obtained from P9 in 1984, during the asymptomatic stage of infection (Fig. 4A). In comparison, there was a more severe depletion of $CD4^+$ T lymphocytes in tissues infected with the HIV-1 Nef variants that were obtained at later stages of infection and that showed higher activity in down-modulation of CD4. The significant correlation between Nef-mediated CD4 down-regulation and the severity of $CD4^+$ T-cell depletion was confirmed using the HIV-1 forms containing the LTNP4/NA7 *nef* alleles ($P = 0.01$; Fig. 4B). Together, the results obtained with NL4-3 and the P9 and LTNP4/NA7 *nef* variants consistently indicate that Nef-mediated CD4 down-regulation correlates with $CD4^+$ T-cell depletion in HLT.

DISCUSSION

Our results demonstrate a strong correlation between the ability of Nef to down-modulate CD4, the efficiency of HIV-1

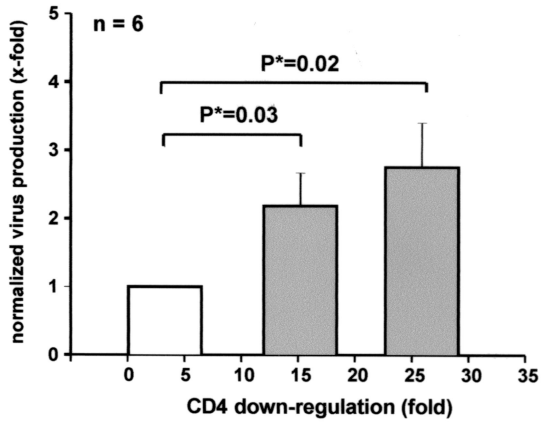

FIG. 3. CD4 down-regulation by primary *nef* alleles correlates with the enhancement of HIV-1 replication in HLT ex vivo. Cumulative p24 production over 11 to 13 days of infection with NL4-3 variants containing *nef* genes obtained from P9 in the course of disease progression plotted against the activity of these *nef* alleles in CD4 down-regulation. Data on viral replication were normalized for production of NL4-3 containing *nef* alleles isolated from P9 in 1984 during the asymptomatic phase of infection (normalized to 1).

replication, and the severity of $CD4^+$ T-cell depletion in HLT ex vivo. Recently, it has been shown that *nef* alleles obtained during or after progression to AIDS are frequently more active in down-modulation of CD4 than those obtained at the asymptomatic stage of infection (4). Taken together, these data imply that the emergence of such Nef variants would be associated with enhanced virus production and greater loss of $CD4^+$ T cells in lymphoid tissues. Thus, altered Nef function likely contributes to the rapid breakdown of the immune system during late stages of HIV-1 infection and could be of particular importance for patients progressing to AIDS in the absence of HIV-1 variants with an expanded coreceptor tropism.

nef alleles defective in CD4 down-regulation and in stimulating viral replication but active in enhancing virion infectivity and in MHC-I down-regulation have been detected in LTNP4 (4, 22) and another long-term survivor of HIV-1 infection (3). Apparently, impaired Nef function in CD4 down-regulation and, accordingly, inefficient viral replication in HLT ex vivo is associated with low viral RNA loads and the absence of disease in HIV-1-infected individuals. The conclusion that Nef-mediated CD4 down-regulation plays an important role in vivo is in agreement with recent findings with the SIV/macaque model (16).

It is beyond the scope of this study to clarify the exact mechanistic link between increased viral replication and CD4 down-modulation. However, concordant with previous results on SIV Nef (16), the present study indicates that the surfaces in Nef involved in CD4 down-regulation and the enhancement of replication are largely overlapping, if not identical. Common molecular interactions of Nef with cellular factors might underlie these two functions. Nonetheless, convincing evidence for a direct mechanistic link is still missing. Clearly, the effect of Nef on virion infectivity is not dependent on the presence of CD4 in the producer cells. Furthermore, the enhancement of HIV-1 infectivity for MAGI cells by Nef did not correlate with the efficiency of viral replication in PBMC or in ex vivo infected HLT. These data indicate that the effect of Nef on virion infectivity does not account for the enhanced replication kinetics in primary T cells. As far as direct effect of CD4 down-modulation on production of virions is concerned, it does not explain why enhanced replication kinetics of *nef*-open HIV are predominantly observed when PBMC are infected immediately after isolation and stimulated several days later but not in prestimulated lymphocyte cultures and in most immortalized T-cell lines (20, 24, 29).

Various data suggest that the major effect of Nef on viral replication is due to lymphoid cell activation (1, 26, 28). Nef affects several aspects of T-cell receptor function. SIVmac Nef down-modulates CD3 from the cell surface, whereas HIV-1 Nef blocks a proximal signaling event (15). Nef uses similar surfaces to down-modulate cell surface expression of both CD4 and CD28 (31). Swigut et al. have proposed that the concerted effect of Nef on CD3, CD4, and CD28 function and the simultaneous activation of downstream effectors in signaling pathways might allow Nef to cause T-cell activation uncoupled from the normal antigen-specific T-cell receptor signaling (31). Thus, these Nef functions might be required simultaneously to increase viral spread. This hypothesis could help to explain why CD4 and CD28 down-regulation and the enhancement of viral replication have evolved to require similar surfaces of the Nef protein, thus becoming mechanistically linked.

In summary, we demonstrate that effective HIV-1 production in HLT ex vivo correlates with CD4 down-regulation but not with increased virion infectivity. *nef* alleles defective in CD4 down-regulation caused only mild $CD4^+$ T-cell depletion in HLT. The fact that such *nef* alleles were derived from long-term nonprogressors of HIV-1 infection (3, 4, 13, 14, 22) suggests that Nef-mediated CD4 down-regulation is important for the full pathogenic potential of HIV-1. Further studies are required to clarify the exact molecular mechanisms by which CD4 down-regulation and viral replication are linked and to elucidate the contribution of altered Nef function to the rapid

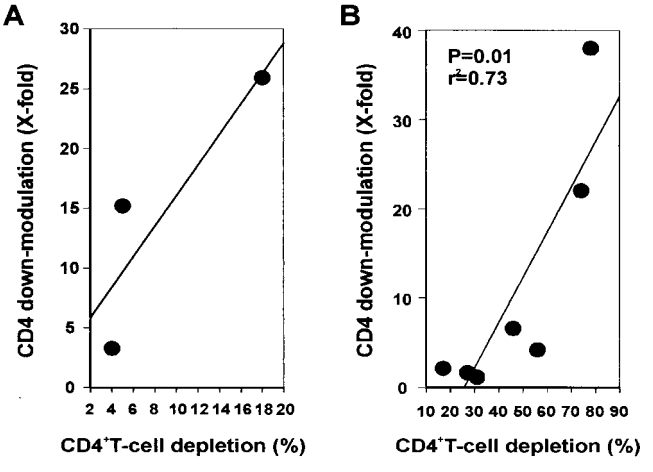

FIG. 4. Correlation between down-regulation of CD4 in vitro and $CD4^+$ T-cell depletion in HIV-1-infected HLT ex vivo. Tissues from two donors were infected with NL4-3 variants containing sequential *nef* alleles drawn from P9 (A) or LTNP4/NA7 *nef* alleles or a deletion in *nef* (B).

destruction of the host immune system at later stages of HIV-1 infection.

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